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(54) Title: STEM CELL INHIBITORS

(57) Abstract

A polypeptide, capable of interacting with haemopoietic stem cells so as to protect them against the cell cycle specific cytotoxic drugs used in cancer chemotherapy, is isolated from macrophage cells of bone marrow origin.

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TITLE: STEM CELL INHIBITORS

THIS INVENTION relates to stem cell inhibitors and is particularly concerned with improvements in the management of cancer chemotherapy.

It is well-known that medullary aplasia represents a limiting factor for the clinical use of cytotoxic drugs which are active in cycling cells during chemotherapy of cancer. It has been recognised for many years that existing methods of chemotherapy could be improved if it were possible to protect the haemopoietic stem cells during treatment with the cytotoxic drug but, in spite of extensive research in this area, no suitably specific inhibitory agent from a readily amenable source has previously been discovered. One of the reasons for this lack of progress is believed to be the difficulty in developing a suitable *in vitro* assay to monitor stem cell regulation.

We have now been able to isolate and identify a proteinaceous substance, obtainable from certain macrophage cell lines, that has the ability to interact with haemopoietic stem cells so as to protect them against the cell cycle specific cytotoxic drugs used in cancer chemotherapy.

Accordingly, the present invention provides a haemopoietic stem cell inhibitor characterised by the following properties:

1. The inhibitor activity is sensitive to degradation with trypsin and the inhibitor is therefore proteinaceous.

2. The inhibitor when partially purified by treatment on an anion-exchanger shows a molecular weight range 45-60 Kd on a molecular weight analysis resin.

3. The inhibitor, when purified to a single band on polyacrylamide gel electrophoresis, under reducing conditions, shows a molecular weight range of 8-10 Kd and a slightly higher molecular weight range under non-reducing conditions.

4. The inhibitor is heat-stable as follows:

(i) Inhibitory activity is retained after heating for 1 hour at 37°C, 55°C and 75°C.

(ii) Inhibitory activity is retained after heating for 10 minutes at 100°C.

5. The inhibitor binds to anion-exchangers and can be eluted with a salt gradient at between 0.26 and 0.28 molar NaCl.

6. The inhibitor binds to a Heparin Sepharose affinity chromatography resin and can be eluted from the resin with one molar sodium chloride buffer.

7. The inhibitor binds avidly to Blue Sepharose affinity chromatography resin and can be eluted from the resin with 5M magnesium chloride.

8. Cycling stem cells, when treated with the partially purified inhibitor, become resistant to the action of cytosine arabinoside. If the treated stem cells are then washed with buffered saline to remove the cytotoxic drug and inhibitor, the surviving stem cells proliferate in culture normally.

The isolation and identification of the inhibitor has been facilitated by the development, for the first time, of an in vitro assay to monitor stem cell regulators. Cycling progenitor cells (more mature cells, not stem cells) are unaffected by the inhibitor. Direct addition of the purified inhibitor to this assay results in inhibition of macroscopic colony development but not on other colonies in the assay.

The in vitro stem cell (CFU-A) assay

For the detection of stem cells in vitro 10^4 bone marrow cells in 4 ml supplemented alpha-modified minimal essential medium (MEM) containing 25% foetal calf or horse serum and 0.3% agar were seeded on top of an underlayer of the same medium containing 0.6% agar, 10% L929 cell conditioned medium (L929 CM, a source of the growth factor CSF-1) and 10% AF1-19T cell conditioned medium (AF1-19T CM) (a source of the growth factor GM-CSF and other uncharacterised stem cell growth factors) in a 6 cm petri dish. Cultures were incubated at 37°C in a fully humidified atmosphere of 10% CO_2, 5%, O_2, 85% N_2 for 11 days. Colonies were stained INT 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride hydrate overnight. Whilst there are colonies in the CFU-A assay with a diameter less than 2 mm, we have chosen this value as a useful cut-off point after preliminary experiments were performed using cytosine arabinoside. We found that within individual dishes, colonies with a diameter <2 mm were mainly derived from cells in cycle, whereas colonies >2 mm were found to be derived from minimally proliferating cells. Only colonies with diameters >2 mm were scored in these assays.

The inhibitor of the present invention is obtainable from several macrophage cell lines. Interest centres initially upon the various known macrophage cell lines of bone marrow origin, typically murine bone marrow as this represents a major source of such macrophage cell lines. Our screening of a population of such cell lines has identified at least two known cell lines capable of producing our inhibitor. In the Examples illustrating this invention, we describe the isolation from one such known macrophage cell line.

As an alternative to the use of known macrophage cell lines, it is also possible to produce suitable macrophage cell lines by the transformation of bone marrow cells with a retrovirus transforming agent.

The inhibitor of the invention can be produced quite simply from the inhibitor-producing macrophage cell line by cultivating the cell line in an appropriate growth medium under conventional conditions, e.g. 37°C, the use of aerobic conditions with 5% v/v carbon dioxide in air provides an ideal growth environment, until the cell concentration is approximately 10^6 per ml. At this stage, the still growing cells are separated from the growth medium, e.g. by centrifugation or preferably membrane filtration and the inhibitor can then be recovered from the supernatant. In order to demonstrate

inhibitor activity, it is desirable that the supernatant first be concentrated, e.g. using membrane dialysis to give a concentration of about 20-fold. The inhibitor can then be isolated from the concentrated supernatant by chromatographic procedures.

A first step of purification may include passing the concentrated supernatant over an anion-exchange resin and eluting a fraction using a 0.25-0.30M NaCl solution.

A second step of purification may include passing the product from the first step over a Sepharose-Heparin column and eluting a fraction using NaCl solution of molarity at least 1M.

A third step of purification may include passing the product from the second step over Sepharose-Blue and eluting a fragment using $MgCl_2$ solution of molarity at least 3M. This elution gives a product that shows a single band on polyacrylamide gel electrophoresis under reducing conditions.

While the main application of the inhibitor of the invention is in clinical practice, as will be described in more detail below, the inhibitor or immunogenically active fragment thereof can also be used as an immunogen to raise antibodies that will recognise part or all of the inhibitor, by immunising a host animal and recovering from the host animal antibodies

or antibody producing cells. Such antibodies can be prepared e.g. in rabbits where polyclonal antibodies can be recovered from the serum of the rabbit or can be used as immunogens in mice for the production of monoclonal antibodies by conventional techniques.

The present invention also extends to DNA sequences encoding the inhibitor of the invention. Such DNA is of interest in the production of the inhibitor by recombinant DNA techniques. Such techniques can involve two different approaches. Both approaches require, as a first step, the production of a gene library from the messenger RNA of the macrophage cell line that naturally produces the inhibitor. This involves the production of a complementary DNA expressible in a bacterial or other host cells.

Having produced the cDNA library, a first approach involves the production and use of DNA probes. Limited sequence analysis of the inhibitor will permit the synthesis of oligonucleotides that can be used to probe the DNA library to identify those cDNA's in the library that hybridise with the probe and so permits the identification of the messenger RNA encoding the whole inhibitor. As an alternative to probing a gene library derived from the macrophage cell, a gene library of human origin can be probed to identify and isolate a DNA

encoding an inhibitor of human origin which is expressable, using techniques now well-known, in a host cell system.

Our preliminary investigation of the biological properties of the inhibitor indicate that its inhibitory effect upon cycling stem cells is reversible. The majority of chemotherapeutic agents used for cancer chemotherapy have a relatively short in vivo half-life, usually less than 24 hours so that the inhibitory effect of the inhibitor of the invention is maintained for at least the major proportion of the effective time during which the chemotherapeutic agent is active in vivo. Our expectation is that the normal physiological mechanisms within the body would limit the effective duration of activity of the inhibitor in relation to the cycling stem cells.

In clinical application, it is desirable to target the inhibitor of the invention to the blood-forming tissue. This targetting can be achieved by injecting the inhibitor, normally by infusion or bolus intravenous administration and the present invention extends to pharmaceutical compositions containing the inhibitor and appropriate diluents or carriers suitable for such parenteral administration.

The interest in the inhibitor of this invention is not restricted to stem cells specific to the haemopoietic system but extends to other stem cells e.g. epithelial stem cells, making the inhibitor of interest not only in relation to alleviating the side effects of cytotoxic drug therapy on bone marrow cells but also in the treatment of solid tumours. The inhibitor is also of interest in the treatment of leukaemia where leukaemic bone marrow cells are treated in vitro or in vivo, with inhibitor so that proliferation of normal stem cells is prevented and the proliferating leukaemic cells can then be treated with a cytotoxic agent.

The invention will now be further illustrated in the following Examples.

EXAMPLE

a) Cell line selected for inhibitor isolation and purification

The widely available J774.2 was used. This is as described in J. Immunol. (1975) 114, 898

Cancer Res. (1977) 37, 546

Selection for serum-free growth in spinner culture

J774.2 cells were originally growing in a

modified Eagles medium supplemented with 10% foetal calf serum. The cells were subcultured into the medium:

Dulbeccos x 10	50 ml
SF12 x 10	50 ml
Glutamine 200mM	10 ml
Sodium pyruvate 100mM	5 ml
distilled water	840 ml
Nutridoma SP	10 ml
Sodium bicarbonate 7.5%	41 ml

Nutridoma SP from Boehringer, Lewes, Sussex, Cat. No. 1011375 : other reagents from Flow Laboratories UK and Gibco-Biocult UK.

The 774.2 cells were subcultured into the above medium with added foetal calf serum (5%) for one week, with a further subculture at three days in the same medium. Cells were further subcultured into medium plus 2.5% foetal calf serum for a week, then into 1% foetal calf serum for a further week until finally all serum was removed. At this stage the cells were subcultured every two days, allowed to grow to a concentration of 8×10^5/ml and diluted to 2×10^5/ml at subculture. Cells were then transferred to spinner culture and acclimatised to growth in suspension, subcultering every two days as described above. This cell line designated J774.2(S) and capable of

growth in serum free suspension culture was then used for inhibitor production.

b) Growth of cells in large scale culture and processing of conditioned medium for purification of inhibitor.

500 ml (8 x 10^5/ml) J774.2(S) were seeded into large fermentors (Techne, 7L) containing 1500 ml of medium. Stirred fermentors were gassed with 5% CO_2 in air, stirred at 20 rpm at 37°C for 2 days, then supplemented with 6L medium and allowed to continue to grow for 3 days.

Undiluted medium from 8 x 10^9 cells is separated from the cells using a Millipore Pellicon-Casette system with a 0.45u microporous membrane. The medium minus cells is then concentrated inthe same apparatus using a 10K cut-off membrane to a final volume of about 400 ml. This concentrate is then processed as described below for biochemical purification.

Biochemical purification

Stage 1. The 400 ml concentrate is desalted on a G-25 HR16/50 (Pharmacia) in 0.02M Tris-HCl pH 7.6, applied to the anion-exchange Mono Q HR10/10 column (Pharmacia, FPLC system) and finally eluted with a salt gradient (0 to 1M

NaCl) in the same buffer. The active fractions of inhibitor elute between 0.26 and 0.28M NaCl.

Stage 2. The impure inhibitor from Stage 1 is applied directly to a Sepharose-Heparin (Pharmacia) column, HR10/10. The inhibitor binds to the resin and the majority of proteins (more than 90%) can be washed off the resin using 0.1M NaCl-0.02M Tris HCl, pH 7.6. The inhibitor is thn eluted from the resin in 1M NaCl-0.02M Tris HCl, pH 7.6.

Stage 3. The partially purified inhibitor from Stage 2 is applied directly to a Blue Sepharose (Pharmacia) column HR5/5 without desalting. The column is then washed with the buffer used to elute the inhibitor in Stage 2, to remove other protein. The purified inhibitor, which binds strongly, is eluted with 5M $MgCl_2$-0.02M Tris-HCl pH 7.6

Other biochemical characteristics of the inhibitor include:

a) Heat stability.

b) It is stable at 37°C , 55°C, 75°C for 1 hour.

Molecular size

The purified inhibitor can be seen as a single band of molecular weight 8-10Kd on polyacrylamide gels under reducing conditions. When the inhibitor is applied to polyacrylamide gel electrophoresis under non-reducing conditions, it can be seen to be of slightly higher molecular weight. These observations suggest that the inhibitor is a single chain polypeptide with internal disulphide bonds affecting electrophoretic mobility.

Biological characteristics of the inhibitor

Reversibly triggers multipotential stem cells (CFU-A, as mentioned in the in vitro assay description) out of cell cycle.

Bone marrow cells were incubated in paired tubes containing 5×10^6 cells in 1 ml Fischer's medium supplemented with 20% horse serum. The inhibitor or alpha-MEM was added to each tube and Fischer's medium was added to control tubes. The mixtures were incubated at $37^{\circ}C$ for 5 hours (inhibition assays). For the last 60 minutes of the incubation $10^{-3}M$ cytosine arabinoside was added to one tube and an equal volume of medium to the other tube. Cells were then washed twice before being

assayed in the CFU-A assay as described above. The inhibitor was found to reduce the number of stem cells in cycle from an average of 30% to an average of about 3%. The untreated cells were killed by the cytotoxic drug treatment in contrast to the inhibitor treated stem cells. Impure preparations of the inhibitor also reversibly trigger multipotential stem cells out of cycle when assayed *in vivo* using the CFU-S assay described in the literature.

CLAIMS

1. A haemopoietic stem cell inhibitor characterised by the following properties:

1. The inhibitor activity is sensitive to degradation with trypsin and the inhibitor is therefore proteinaceous.

2. The inhibitor when partially purified by treatment on an anion-exchanger shows a molecular weight range 45-60 Kd on a molecular weight analysis resin.

3. The inhibitor, when purified to a single band on polyacrylamide gel electrophoresis, under reducing conditions, shows a molecular weight range of 8-10 Kd and a slightly higher molecular weight range under non-reducing conditions.

4. The inhibitor is heat-stable as follows:

(i) Inhibitory activity is retained after heating for 1 hour at 37°C, 55°C and 75°C.

(ii) Inhibitory activity is retained after heating for 10 minutes at 100°C.

5. The inhibitor binds to anion-exchangers and can be eluted with a salt gradient at between 0.26 and 0.28 molar NaCl.

6. The inhibitor binds to a Heparin Sepharose affinity chromatography resin and can be eluted from the resin with one molar sodium chloride buffer.

7. The inhibitor binds avidly to Blue Sepharose affinity chromatography resin and can be eluted from the resin with 5M magnesium chloride.

8. Cycling stem cells, when treated with the partially purified inhibitor, become resistant to the action of cytosine arabinoside. If the treated stem cells are then washed with buffered saline to remove the cytotoxic drug and inhibitor, the surviving stem cells proliferate in culture normally.

2. An inhibitor according to claim 1 obtained from macrophage J774.2 cells.

3. A pharmaceutical composition comprising an inhibitor according to claim 1 or 2 together with a pharmaceutically acceptable carrier or diluent.

4. A composition according to claim 3 for parenteral administration.

5. An antibody to an inhibitor according to claim 1 or 2.

6. A method of raising an antibody which comprises immunising a host animal with an inhibitor according to claim 1 or 2 or with an immunogenically active fragment of the inhibitor and recovering antibody or antibody producing cells from the host animal.

7. Synthetic DNA including DNA encoding the inhibitor according to claim 1 or 2 expressable in a host cell.

8. A method of isolating and purifying an inhibitor as defined in claim 1 which includes the steps of:

1. growing an inhibitor producing macrophage cell line of bone marrow origin in a growth medium to produce a supernatant containing inhibitor;

2. contacting the supernatant with an anion-exchanger and eluting the anion-exchanger with

0.25-0.30M NaCl solution to give a first eluant containing the inhibitor.

3. contacting the first eluant with Sepharose-Heparin and eluting the Sepharose-Heparin with NaCl solution of molarity at least 1M to give a second eluant containing the inhibitor.

4. contacting the second eluant with Sepharose-Blue and eluting the Sepharose-Blue with $MgCl_2$ solution of molarity at least 3M to give a third eluant containing the inhibitor.

9. An inhibitor according to claim 1 or 2 for use in a method of treatment of the human or animal body by therapy.

10. A method of treating a patient receiving a cell cycle specific cytotoxic drug which includes administration of an inhibitor according to claim 1 or 2 so as to protect the patient's haemopoietic stem cells against damage by the cytotoxic drug.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00396

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: A 61 K 37/02, C 07 K 15/00, C 12 N 15/00, C 07 K 3/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 P, A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X,P	Chemical Abstracts, vol. 109, 1988, (Columbus, Ohio, US), I.B. Pragnell et al.: "The effect of stem cell proliferation regulators demonstrated with an in vitro assay", see page 344, abstract 89048m, & Blood 1988, 72(1), 196-201 --	1,8
X,P	Biological Abstracts, Reviews-Reports-Meetings, vol. 35, 1988, no. 106933, E.G. Wright et al.: "The effect of stem cell proliferation regulators demonstrated with an in-vitro assay", & Experimental Hematology, 1988, vol. 16, no. 6, page 473 see title and terms --	1,8

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10th August 1989	14.08.89
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	T.K. WILLIS

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Biological Abstracts, Reviews-Reports-Meetings, vol. 33, 1987, no. 61402, A. Janowska-Wieczorek et al.: "Protection of human hematopoietic stem cells from tubercidin toxicity by inhibitors of nucleoside transport", & Proc. Am. Assoc. Cancer Res. Annu. Meet. 1987, vol. 28, no. 0, page 409 see title and terms --	1
A	Chemical Abstracts, vol. 96, 1982, (Columbus, Ohio, US), M. Guigon et al.: "Protection of mice against lethal doses of 1beta-D-arabinofuranosylcytosine by pluripotent stem cell inhibitors", see page 36, abstract no. 115633h, & Cancer Res. 1982, 42(2), 638-41 ---	1

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☒ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☒ Claim numbers 10, because they relate to subject matter not required to be searched by this Authority, namely:

See PCT-Rule 39.1(IV); methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.